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13. ABSTRACT *(Maximum 200 words)*

Mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) has been identified as a putative tumor suppressor gene in both breast and liver cancers due to identification of loss of an allele at the M6P/IGF2R gene locus accompanied by somatic mutations in the remaining allele. Loss of M6P/IGF2R function is potentially complex because the receptor has a number of physiological roles including: 1) trafficking of mannose 6-phosphorylated lysosomal enzymes to endosomal compartments; 2) playing a role in proteolytic activation of transforming growth factor β, a negative growth regulator and; targeting the potent mitogen IGF2 for degradation. Although genetic evidence is strong, there is no direct evidence to support the hypothesis that M6P/IGF2R is a tumor suppressor. In this investigation, transfection studies demonstrated that cells overexpressing wildtype (wt) M6P/IGF2R or a mutant deficient in M6P protein binding M6P*def*R, showed reduced IGF2-stimulated growth when compared to cells transfected with a negative control or a mutant M6P/IGF2R unable to bind IGF2 (IGF2*def*R). Further, when IGF1- and IGF2-stimulated growth were compared, only IGF2-stimulated growth was suppressed in cells overexpressing wt M6P/IGF2R or M6P*def*R, suggesting that M6P/IGF2, as an IGF2 antagonist, is important in tumor suppression.

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Stacey Eubank 29 July 1999

PI - Signature Date

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Introduction

The focus of this investigation is to provide functional data to support recent genetic evidence that *mannose 6-phosphate insulin-like growth factor receptor* (*M6P/IGF2R*) may be a tumor suppressor gene in breast cancer. Mutational screening has identified *M6P/IGF2R* as a putative tumor suppressor gene: loss of heterozygosity of one *M6P/IGF2R* allele, accompanied by somatic mutations in the remaining allele has been demonstrated in approximately 70% of liver cancers (1, Yamada, 1997 #613) and 30% of breast cancers (2). Furthermore, somatic mutations in coding region microsatellites have also been detected in a variety of malignancies with the replication error positive phenotype (3, 4, 5, 6).

Physiologically, well-defined functions of M6P/IGF2R include: 1) to facilitate endocytosis of extracellular mannose 6-phosphorylated (M6P) lysosomal proteins (7); 2) to escort newly synthesized M6P proteins from the Golgi to endosomes (8); 3) to play a role in the proteolytic activation of transforming growth factor β (TGFβ), a negative growth regulator (9); and 4) to target insulin-like growth factor 2, a potent mitogenic peptide, for degradation thereby preventing insulin-like growth factor 1 receptor (IGF1R) activation (10). The cellular consequences of loss of M6P/IGF2R function are therefore potentially wide-ranging. It has been hypothesized that loss of M6P/IGF2R function may influence tumor cell growth by promoting tumor invasion through lysosomal enzyme misrouting, decreasing the growth inhibitory activity of TGFβ, and increasing the mitogenic and cell survival activities of IGF2 (11). Although there is some evidence that the role of M6P/IGF2R as an IGF2 antagonist is critical in regulating cellular growth (10, 11, 12, 13), to date, there is no direct evidence to support the hypothesis that M6P/IGF2R function is important for suppression of tumor cell growth. This study addresses the hypothesis that M6P/IGF2R is a tumor suppressor gene in IGF2-sensitive malignancies.

Current Progress

To investigate the negative growth regulatory properties of M6P/IGF2R, the breast cancer cell line MCF7 has been transfected with expression constructs for bovine wildtype M6P/IGF2R and M6P/IGF2R defective in either M6P or IGF2 ligand-binding activities. The bovine M6P/IGF2R cDNA has been utilized because it is well-characterized, and in transfection studies with human cell lines, transfected bovine receptor can be distinguished from endogenous human M6P/IGF2R with species-specific monoclonal antibody. Further, the human and bovine M6P/IGF2R cDNAs share 80% sequence identity (14) and experiments examining the M6P protein sorting properties of the bovine and human M6P/IGF2R demonstrated that there were no functional differences between them (15, 16).

Wildtype bovine M6P/IGF2R and M6P-binding deficient bovine M6P/IGF2R (M6P*def*R) cDNAs were kindly provided by Dr N. Dahms (15). The IGF2-binding deficient bovine M6P/IGF2R (IGF2*def*R) was generated in the Ellis laboratory by a site-directed mutagenesis polymerase chain reaction approach. As a negative control, a frame shift mutation encoding a nonfunctional truncated M6P/IGF2R (D95STOP) was also synthesized. The binding selectivity of these constructs has been confirmed. This project employs these receptor constructs to evaluate the potential role of M6P/IGF2R as a tumor suppressor gene.

Proposed Statement of Work to be completed in months 13 to 24.

1) Establish the growth effects of inducible expression of bovine M6P/IGF2R in MCF7 cells in vitro and investigate these growth effects of ligand binding mutants already established by the laboratory.

2) Develop an animal protocol for the investigation of the phenotypic consequences of M6P/IGF2R in vivo in tumor xenografts. Conduct pilot animal experiments.

3) Subclone the human M6P/IGF2R into a tetracycline inducible expression vector and compare growth effects of human and bovine M6P/IGF2R *in vitro*.

Work completed in months 13 to 24

1) Accomplishments in the first year demonstrate that M6P/IGF2R has a role in cellular proliferation of MCF7 cells in response to IGF2-stimulated growth. Overexpression of wildtype M6P/IGF2R and M6P*def*R (deficient in M6P-binding, but binds IGF2) suppresses IGF2 dependent growth of MCF7 cells when compared to cells expressing IGF2*def*R (deficient in IGF2-binding but binds M6P-proteins) or D95STOP (negative control). However, these experiments do not address the specificity of IGF2 antagonistic growth regulation due to M6P/IGF2R overexpression. Growth response to IGF1 treatment compared to IGF2 treatment is currently being investigated with soft agar assays utilizing MCF7 cells either constitutively or inducibly overexpressing the various M6P/IGF2R constructs. MCF7 cells stably transfected with constitutive expression constructs for wildtype M6P/IGF2R, M6P*def*R, IGF2*def*R and D95STOP are plated in triplicate in 0.3% agar over a 0.6% agar cushion in 6-well dishes. The cells are treated with 10nM IGF1, 10nM IGF2 or no treatment for 2 weeks, changing the media every 3 or 4 days. The number of colonies are determined with an automated colony counter. Preliminary experiments, demonstrated in Figure 1, suggest the specificity of the negative growth regulatory activity of M6P/IGF2R as an IGF2 antagonist. There was no significant difference between the number of colonies formed from cells overexpressing D95STOP or IGF2*def*R treated with either IGF1 or IGF2, but colony numbers of cells overexpressing wt M6P/IGF2R or M6P*def*R indicated suppression of IGF2-stimulated growth when compared to IGF1-

stimulated growth. These experiments are currently being repeated. Further, the clone which inducibly expresses wildtype M6P/IGF2R (iWt1) is also being utilized in soft agar assays. IGF1- and IGF2-stimulated colony growth is being compared in cells treated with doxycycline to inducibly express M6P/IGF2R with cells not doxycycline-treated.

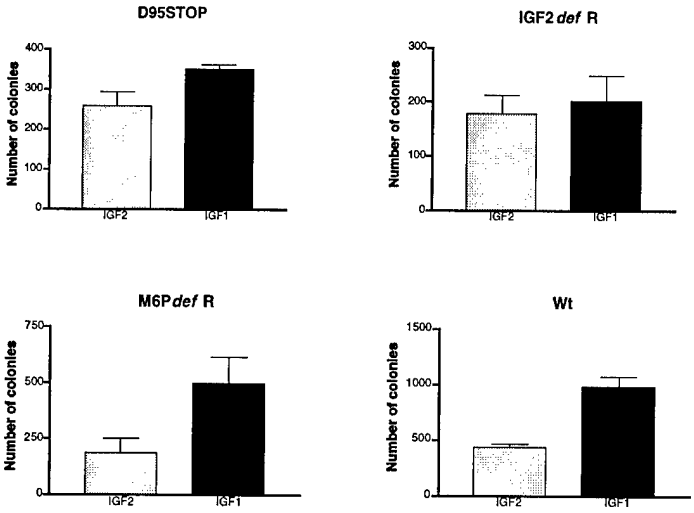

Figure 1. Bar graph showing the number of colonies detected in response to IGF1 and IGF2 stimulated growth for cells overexpressing the various M6P/IGF2R constructs. Cells expressing wildtype M6P/IGF2R or M6P*def*R showed growth suppression in response to IGF2 stimulated growth when compared with IGF1 stimulated growth. This is contrasted with cells overexpressing negative control, D95STOP or IGF2*def*R where growth rates were similar For IGF1 and IGF2 stimulated growth.

Over the past year, several MCF7 clones transfected with the Tet-On inducible gene system have been screened by immunoflourescence and western blot analysis in order to identify clones which inducibly express M6P*def*R (iM6P*def*R1) and IGF2*def*R (iIGF2*def*R1). One clone expressing each of the receptor mutants was identified and Figure 2 shows Western blot analysis of receptor induction with doxycycline. A polyclonal antibody that recognizes both human and transfected mutant bovine M6P/IGF2R allows an assessment of the overexpression of mutant receptor over the endogenous

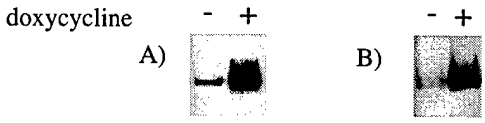

Figure 2. Western blot of whole cell lysates of MCF7 cells that inducibly express mutant bovine M6P/IGF2R A) deficient in binding M6P- proteins (iM6P*def*R1) or B) deficient in IGF2 binding (iIGF2*def*R). Cells were treated with or without 1000ng/ml doxycycline in 10% FBS DMEM media for 24 hours, subject to SDS-PAGE,transferred to nitrocellulose and protein detected by a polyclonal antibody for the induced mutant receptor.

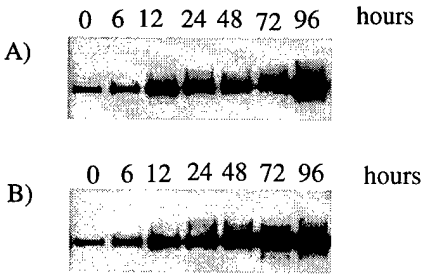

Figure 3. Inducible expression of M6P*def*R in MCF7 cells (M39.1). (A) Protein expression induced with 250ng/ml of doxycycline. (B) Protein expression induced with 500ng/ml of doxycycline in 10% FBS DMEM media for 0 - 96 hours, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The protein was detected by polyclonal antibody for the induced mutant bovine receptor.

human background. Inducible M6P*def*R expression peaks at approximately 24 hours and remains elevated after 96 hours of doxycycline treatment (Figure 3), similar to that seen for inducible expression of wt M6P/IGF2R in iWt1 cells. Inducible IGF2*def*R expression is similarly being characterized.

2) We have developed an animal protocol that has been approved by the Georgetown Unversity Animal Care and Use Committee (GUACUC). The protocol reference number is 98-093 and is entitled "Mannose 6-phosphate/Insulin-like Growth Factor 2 Receptor (M6P/IGF2R) is a Negative Growth Regulator." However, before attempting expensive animal experiments, we decided to investigate other end-points in addition to cellular proliferation, to support the hypothesis that IGF2 antagonistic activity of M6P/IGF2R is important in suppression of tumor cell growth.

IGF2 evokes cellular proliferation and anti-apoptotic activity through IGF1R-mediated activation of downstream signaling molecules (17) including IRS-1 which is phosphorylated on multiple tyrosine residues in response to IGF1R activation (17). We therefore investigated IRS-1 tyrosine phosphorylation in response to IGF1 or IGF2 treatment in cells inducibly expressing the wildtype receptor. We hypothesize that overexpression of wt M6P/IGF2R will increase IGF2 degradation and result in reduced IGF1R activation and IRS-1 phosphorylation in response to IGF2 stimulation but not IGF1 stimulation. iWt1 cells were plated in 6cm dishes and treated with or without doxycycline in 10% FBS DMEM media for 48 hours. After serum starvation for 24 hours, the cells were treated with or without 40nM IGF1 or IGF2 for various time points, lysed, and cell extracts were subjected to immunoprecipitation (IP) with IRS-1 antibody followed by Western blotting with an antiphosphotyrosine monoclonal antibody. Figure 4 is representative of 4 IRS-1 IP experiments demonstrating no reduction of IRS-1 activation in cells overexpressing wt M6P/IGF2R in response to either IGF treatment up to 1 hour. We are currently repeating these experiments with extended time points and will include iM6P*def*R1 and iIGF2*def*R1 clones that inducibly express the mutant bovine M6P/IGF2R constructs. It must be noted that 40nM IGF concentration is used in experiments with the MCF7 clones transfected with the Tet-On gene activation system because lower IGF concentrations elicited little or no response as detected in IRS-1 IP experiments with various IGF concentrations (Figure 5) or in tetrozolium salt colorimetric growth assays (data not shown). This may be because the parent MCF7 cells into which the Tet-On regulator plasmid was transfected were obtained from Dr Doug Yee and not the Lombardi Cancer Center (LCC) MCF7 cell pool used in previous transfections which is sensitive to lower IGF concentrations (Figure 5).

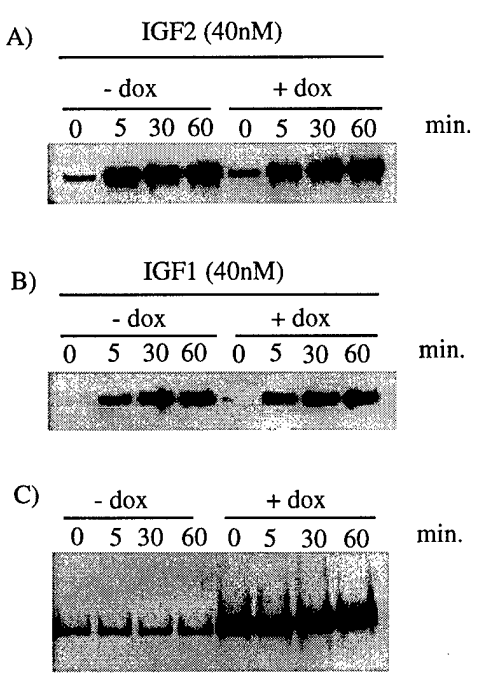

Figure 4. IRS-1 phosphorylation of MCF7 clones inducibly expressing wildtype M6P/IGF2R in response to A) IGF2 or B) IGF2 at the time points indicated. Panel C) is a Western blot demonstrating the level of overexpressed M6P/IGF2R above the endogenous background of human M6P/IGF2R in the transfected MCF7 cells.

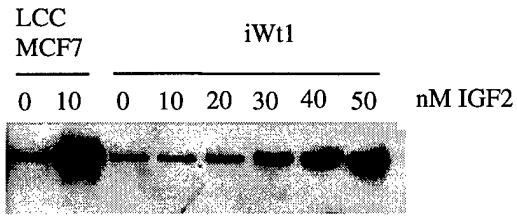

Figure 5. Tyrosine phosphorylation of IRS1 in response to various concentrations of IGF2. Wt 9 cells were treated with IGF2 for 5 minutes. IRS1 was immunoprecipitated from cell lysates, electrophoresed on an SDS-PAGE gel, transferred to nitocellulose and detected with a monoclonal phosphotyrosine antibody.

3) Expression of human M6P/IGF2R (hM6P/IGF2R) in mammalian cells have been problematic since expression is lost after the one passage of the transfected cells. Subcloning of the human M6P/IGF2R into the inducible expression vector has been unsuccessfully attempted and will be repeated.

Conclusion

Preliminary data from soft agar assays using cells that constitutively overexpress various M6P/IGF2R constructs, indicate that the function of M6P/IGF2R as an IGF2 antagonist is important in suppression of IGF-stimulated MCF7 tumor cell growth. MCF7 cells overexpressing M6P/IGF2R constructs that bind IGF2 (wtM6P/IGF2R and M6P*def*R), demonstrated reduced growth in response to IGF2 stimulation when compared to growth stimulated by IGF1. However, MCF7 cells overexpressing M6P/IGF2R constructs incapable of IGF2 binding showed no growth reduction in response to either IGF growth stimulation.

The panel of clones that inducibly express the various M6P/IGF2R constructs in response to doxycycline treatment will allow further evaluation of the growth regulatory role of M6P/IGF2R in response to IGF2 stimulation and will be invaluable in future animal experiments.

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Appendix I – Key Accomplishments

1. Demonstrated specificity of the negative growth regulatory function of M6P/IGF2R as an IGF2 antagonist.

2. Identified MCF7 clones that inducibly express M6P*def*R and IGF2*def*R, mutant M6P/IGF2R constructs that are deficient in M6P protein binding and IGF2 binding respectively.

Appendix II – Reportable Outcomes

1. MCF7 clones that inducibly express wt M6P/IGF2R and mutant M6P/IGF2R constructs, M6P*def*R and IGF2*def*R.

2. A review enitled "Mannose 6-Phosphate/Insulin-like Growth Factor 2 Receptor, a bona fide Tumor Suppressor Gene or Just a Promising Candidate?" to be published in the January 2000 issue of the Journal of Mammary Neoplasia (see attached).

Mannose 6-Phosphate/Insulin-Like Growth Factor 2 Receptor, a *bona fide* Tumor Suppressor Gene or Just a Promising Candidate?

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Lisa M. Schumaker

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Journal of Mammary Neoplasia

July 1999.

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Abstract

The mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) is considered a "candidate" tumor suppressor gene. This hypothesis has been provoked by the identification of loss of heterozygosity (LOH) at the *M6P/IGF2R* locus on 6q25-27 in breast and liver cancer, accompanied by point mutations in the remaining allele. Somatic mutations in coding region microsatellites have also been described in replication error positive (RER+) tumors of the gastrointestinal tract, endometrium and brain. These genetic data are compelling, but a tumor suppressor gene candidate has to meet functional as well as genetic criteria. This review weighs the evidence and discusses the observations that are necessary to promote *M6P/IGF2R* from candidate to *bona fide* tumor suppressor gene.

Key words:

Mannose 6-phosphate/insulin-like growth factor 2 receptor, tumor suppressor gene, breast cancer, loss of heterozygosity, somatic mutation, microsatellite instability.

Abbreviations:

Cirrh., cirrhosis; DCIS, ductal carcinoma *in situ*; HCC, human hepatocellular carcinoma; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; IGF2, insulin-like growth factor 2; LIF, leukemia inhibitory factor; LOH, loss of heterozygosity; LTGFβ, latent transforming growth factor β; M6P, mannose 6-phophate; M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor 2 receptor; MEF, mouse embryonic fibroblast; MI, microsatellite instability; MS, microsatellite; NSCLC, non-small cell lung carcinoma; RA, retinoic acid; RER+, replication error positive; sM6P/IGF2R, soluble mannose 6-phosphate/insulin-like growth factor 2 receptor; TGFβ, transforming growth factor β; uPA, plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

Introduction

The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), first identified as a lysosomal transport protein, has received increased attention following the discovery of somatic mutations in the *M6P/IGF2R* gene. Loss of one *M6P/IGF2R* allele, accompanied by somatic mutations in the remaining allele, has been demonstrated in liver and breast tumors (1, 2, 3, 4). Furthermore, somatic mutations in coding region microsatellites have been identified in a spectrum of malignancies with the replication error positive (RER+) phenotype (5, 6, 7, 8).

The phenotypic consequences of loss of M6P/IGF2R through somatic mutation are potentially very complex, since M6P/IGF2R has a number of roles in cellular physiology. Established functions include (Figure 1): a) to facilitate the transport of newly synthesized mannose 6-phosphorylated (M6P) lysosomal proteins from the Golgi network to lysosomal compartments (9); b) to facilitate endocytosis of extracellular M6P-tagged lysosomal enzymes (10); c) to play a role in the proteolytic activation of transforming growth factor β (TGFβ), a negative growth regulator of epithelial cells (11); and d) to target insulin-like growth factor 2 (IGF2) for lysosomal degradation, thereby preventing insulin-like growth factor 1 receptor (IGF1R) activation (12). Loss of function mutations in M6P/IGF2R could, therefore, contribute to multi-step carcinogenesis by increasing the mitogenic and cell survival activities of IGF2, decreasing the growth inhibitory activity of TGFβ and by promoting tumor invasion through lysosomal enzyme misrouting. (13). The role of M6P/IGF2R in tumorigenesis may be even more complex than this initial list implies. The list of M6P/IGF2R ligands has been extended in recent years and has become remarkably diverse (Table I).

Function and structural domains of M6P/IGF2R

The *M6P/IGF2R* gene has been localized to 6q25-27 (14) and encodes a 2450-amino acid transmembrane receptor of 275 to 300kD. Approximately 90% of M6P/IGF2R molecules are located in the Golgi and pre-endosomal compartment. Receptors are also present in the plasma membrane. These two cellular pools continuously cycle, internalizing extracellular peptides and participating in Golgi to endosome transfer of M6P proteins (principally lysosomal enzymes) (15). M6P/IGF2R has four major

functional domains: an amino-terminal signal sequence, a large extracytoplasmic domain, a transmembrane domain and a short cytoplasmic domain. Figure 2 diagrams the key site-directed mutations in the bovine M6P/IGF2R that have proved valuable in defining structure/function relationships. While M6P/IGF2R was thought to operate as a monomer, recent data suggest that M6P/IGF2R is capable of dimerization, similar to other transmembrane receptors (16).

The ligand-binding extracytoplasmic domain consists of 15 homologous units, each approximately 147 amino acids in length, with 16-38% sequence identity. The repeats are cysteine-rich and the locations of the cysteine residues are highly conserved among the repeating units (15). M6P/IGF2R binds IGF2 and proteins bearing mannose 6-phosphate residues at distinct sites on the receptor (15, 17). The mannose 6-phosphate binding domain is comprised of amino acids in repeats 3 and 9. Substitutions of arginine to alanine at amino acid 435 in repeat 3 and 1334 in repeat 9 (bovine receptor sequence) dramatically reduces M6P-protein binding (18). The "core" IGF2 binding site is within repeat 11 (19, 20), since a single substitution of isoleucine for threonine at position 1572 completely abolished IGF2 binding to a human repeat 11 minigene construct (21). Our laboratory has recently confirmed this conclusion by demonstrating that a homologous amino acid substitution at residue 1581 in the full-length bovine M6P/IGF2R completely disrupts IGF2 binding (22). Recent evidence suggests that repeat 13 also contributes to high affinity IGF2 binding (23). High affinity binding of M6P-proteins and IGF2 apparently does not occur simultaneously, at least in an efficient manner, perhaps due to steric hindrance or conformational changes that occur upon ligand binding (24, 25).

The cytoplasmic domain contains amino acid motifs that mediate endocytosis and lysosomal enzyme transport (26). Analysis of truncation mutants has revealed a casein kinase II site that is important for regulating lysosomal enzyme transport (27). In addition, mutant receptors containing alanine for tyrosine substitutions in the cytoplasmic domain cause receptor accumulation at the cell membrane, suggesting disruption of an “internalization domain” (26, 28).

A soluble form of M6P/IGF2R (sM6P/IGF2R), lacking the transmembrane and cytoplasmic domains, has been identified in tissue culture medium, serum, urine and amniotic fluids of humans and rats (29, 30, 31, 32). It is debatable whether the soluble M6P/IGF2R is merely a degradation product or has physiological function. Recent evidence indicates the latter; sM6P/IGF2R is able to bind IGF2 and inhibit IGF2-induced DNA synthesis in rat hepatocytes (33). Furthermore, transgenic mice engineered to express sM6P/IGF2R at high levels in the skin and alimentary canal exhibited reduced alimentary tract growth, reduced levels of circulating IGF2 and increased levels of latent TGFβ (LTGF-β) (34).

In addition to lysosomal enzymes, M6P/IGF2R interacts with a number of mannose 6-phosphorylated cytokines, including the angiogenic peptide proliferin (35) and the growth and differentiation factor LIF (leukemia inhibitory factor) (36). Although it has been suggested that M6P/IGF2R binding to these proteins leads to internalization and degradation, the actual significance of these binding complexes has not been determined.

More recently, it has been reported that M6P/IGF2R binds to two further classes of ligands at sites other than those involved in either M6P-protein binding or IGF2 binding. Surprisingly, retinoic acid (RA), a molecule essential for development, cellular metabolism and the regulation of cell proliferation, interacts with M6P/IGF2R. Kang *et al.* used photoaffinity labeling to identify specific binding of RA to M6P/IGF2R in myocytes which express high levels of the receptor (37). The site at which RA binds to M6P/IGF2R has not yet been determined, but neither M6P nor IGF2 inhibited the RA-M6P/IGF2R interaction, suggesting a distinct binding site. RA has been shown to stimulate M6P/IGF2R-mediated internalization of IGF2 and increase lysosomal enzyme sorting. Regulation of M6P/IGF2R may therefore be a further mechanism whereby retinoids regulate cellular proliferation (37).

The urokinase-type plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol (GPI)-anchored membrane protein, also binds to M6P/IGF2R in an M6P-independent manner (38, 39). uPAR is found on the surface of most cell types and has a role in a variety of cellular functions, including cell surface adhesion, migration and extracellular proteolysis, as well as activation of the negative growth regulator TGFβ (40). M6P/IGF2R is thought to stabilize a complex between uPAR, its ligand urokinase-

type plasminogen activator (uPA), and plasminogen. Plasmin is then generated, facilitating the proteolytic activation of mannose 6-phosphorylated latent TGFβ bound to M6P/IGF2R (38, 39, 41, 42). *In vitro* binding assays using recombinant-tagged M6P/IGF2R suggest that like retinoic acid, M6P-proteins and IGF2, uPAR has a distinct binding site (39).

Although the exact functional consequences of the various interactions that occur with M6P/IGF2R are uncertain, the increasing number of M6P/IGF2R ligands listed in Table I serves to emphasize multiple potential links between M6P/IGF2R and tumor pathophysiology. The design of experiments that probe the role of M6P/IGF2R in tumor suppression might best be obtained in systems in which at least one of these ligands were critical for tumor development.

M6P/IGF2R in development

Nissley *et al.* used quantitative immunoblotting and Northern blotting to determine that M6P/IGF2R is expressed in most rat fetal tissues, with the highest levels of receptor expression in the fetal heart. High expression levels in cardiac tissue decline rapidly in early postnatal life (43), suggesting a critical role in cardiac development. This hypothesis was confirmed by the finding that perinatal death in *m6p/igf2r* knockout mice was due to cardiac overgrowth. Fetuses null for *m6p/igf2r* also display generalized increased growth and are, on average, 30% bigger than normal littermates. Evidence suggests that the overgrowth phenotype in M6P/IGF2R deficient mice is most likely the result of unchecked IGF2 stimulation, rather than loss of TGFβ activation, or improper lysosomal enzyme sorting: *m6p/igf2r* null mice have elevated levels of IGF2 (44) and, importantly, the fatal effects of M6P/IGF2R absence on the neonatal mouse were reversed in an *igf2* null background (45). These data underscore the conclusion that M6P/IGF2R regulates the availability of IGF2 for IGF1R tyrosine kinase activation (12).

Signal transduction

The discovery that the cation-independent mannose 6-phosphate receptor bound IGF2 led to speculation that M6P/IGF2R was involved in growth factor signal transduction. However, M6P/IGF2R does not possess tyrosine kinase activity or other clear-cut signal transduction capability. Nonetheless, early observation did suggest that IGF2 induced proliferation of rat metanephrons and stimulated DNA synthesis in BALB/c 3T3 cells through M6P/IGF2R (46, 47). In 1991, Okamoto *et al.* suggested these

effects were mediated by a G-protein interaction with the M6P/IGF2R cytoplasmic domain (48). More recent studies have not confirmed these results (49, 50). Furthermore, our laboratory has observed that IGF2 mutants that bind M6P/IGF2R, but not IGF1R, do not induce proliferation of MCF7 breast cancer cells (12). It is now therefore generally accepted that M6P/IGF2R does not function in transmembrane signaling or mitogenesis. Early observations can probably be explained through activation of IGF1R, or perhaps the insulin receptor, since IGF2 has recently been shown to effectively activate insulin receptor signaling (51, 52).

Loss of heterozygosity and point mutations in *M6P/IGF2R*.

Table II lists publications on the frequency of *M6P/IGF2R* LOH and mutation in different tumor types. LOH at the *M6P/IGF2R* locus has been observed in breast carcinoma (1, 53), hepatocellular carcinoma (HCC) (2, 4, 54), gastrointestinal cancer (5, 6), and non-small cell lung carcinomas (55). An isolated finding of LOH at this locus does not, however, prove that chromosomal loss was driven by presence of the *M6P/IGF2R* gene. Identification of putative loss-of-function mutations in the remaining allele in some cases of HCC (3, 4) strengthened the case that loss of M6P/IGF2R was critical. LOH was also seen in premalignant lesions adjacent to HCC cells with *M6P/IGF2R* loss, suggesting that disruption of *M6P/IGF2R* can be an early event in HCC (4).

M6P/IGF2R LOH and point mutations have also been described in breast cancer. LOH was demonstrated in 33% (7 of 21) of informative invasive breast cancer cases and 26% (5 of 19) of informative ductal carcinoma *in situ* (DCIS) cases (1). All of the *in situ* cases that displayed *M6P/IGF2R* LOH were high grade. It has therefore been suggested that *M6P/IGF2R* LOH and mutation preferentially occur during the preinvasive phase of breast cancer development. Mutation screening of the DCIS samples revealed missense mutations in 2 of the 5 tumors examined. These mutations were detected in repeat 10 and in the cytoplasmic domain of the receptor (1). It was proposed that the mutation in repeat 10 could have disrupted IGF2 binding due to the proximity of the IGF2 binding site located in repeat 11. It was also speculated that the cytoplasmic domain mutation had disrupted receptor trafficking. However, these predictions have not been confirmed in molecular and functional studies. A second publication

supports the proposition that loss of M6P/IGF2R function occurs in high grade preinvasive breast cancer. Chappell *et al.* studied 40 non-palpable, well-differentiated invasive breast cancers (detected by mammography) and 22 cases of DCIS for LOH at the *M6P/IGF2R* locus. A total of 53 cases were informative. None of the early well-differentiated invasive cancers demonstrated LOH at the *M6P/IGF2R* locus but 4 of 18 informative DCIS cases were positive for LOH and 3 of these 4 cases were high grade (53).

Somatic mutations of M6P/IGF2R have recently been identified in two other common malignancies. In prostate cancer, mutations in *M6P/IGF2R* in 5 out of 18 samples were described (56). In addition, the complete coding sequence of *M6P/IGF2R* has been screened in lung cancer cell lines with the description of a point mutation in a lung adenocarcinoma-derived cell line (57). The mutation in question was detected in exon 40 where previous mutations have been identified in gastric and liver tumors (3, 5), suggesting a possible mutational hot spot. Kong *et al* used two newly described polymorphisms in the 3' untranslated region of the *M6P/IGF2R* in a polymerase chain reaction to identify LOH mutations in non-small cell lung carcinoma (NSCLC) for LOH. 25 of 35 samples (71%) were informative, 13 of which had LOH at the *M6P/IGF2R* locus. Approximately half of the cases with LOH also had point mutations resulting in significant amino acid substitutions and deletions (55).

Finally, there have been reports of 6q loss in ovarian cancer (58, 59, 60). In one report, *M6P/IGF2R* somatic mutations were described in the remaining allele (58). However, a study that fine-mapped deleted regions on the distal end of chromosome 6q found that *M6P/IGF2R* was not always within the common region of loss. These data suggest that a conclusion that *M6P/IGF2R* is "the" 6q tumor suppressor gene in ovarian cancer is premature (61).

M6P/IGF2R coding region microsatellite instability

Cells which harbor mutations in mismatch repair genes are termed replication error positive (RER+). The RER+ phenotype is expressed as nucleotide insertions or deletions in repetitive nucleotide sequences (microsatellites). When microsatellites occur within coding sequence, gain or loss of nucleotides causes frame-shift and premature translational arrest. M6P/IGF2R has several coding region

microsatellites that are targeted for mutation in RER+ cancers. In a study of 92 RER+ gastrointestinal tumors, 12 had microsatellite mutations within the coding region of the *M6P/IGF2R* gene; 11 were identified in the same poly $(G)_8$ microsatellite (5). Oliveira and colleagues also recently published information demonstrating poly $(G)_8$ microsatellite mutation in 7 of 28 RER+ gastric carcinomas (8). Unlike many of the missense mutations described in conjunction with LOH, microsatellite mutations are definitively associated with loss of function since coding sequence is lost. TGFβ1 type II receptor (TGFβIIR) coding region microsatellites are also subject to mutation in RER+ cancers, and it is interesting to note that *M6P/IGF2R* and *TGFβIIR* are rarely mutated in the same cancer. This suggests that inactivation of M6P/IGF2R and TGFβIIR disrupts a common pathway for tumor progression, which can be taken as indirect evidence for the role of M6P/IGF2R in down-regulating TGFβ signaling during tumorigenesis. Similar conclusions have been made by Ouyang *et al.*, who studied *M6P/IGF2R* and *TGFβIIR* microsatellite mutations in RER+ gastric and colon adenocarcinomas (6). Finally, it has recently been observed that *M6P/IGF2R* is targeted for mutation in RER+ gliomas (7) and endometrial cancers (6, 62). This suggests that M6P/IGF2R loss may be a general feature of RER+ tumors. Although only 8% of breast cancers are reported to be RER+ (63), the largest subset of these tumors (40%) is of the invasive lobular type (64). Therefore, *M6P/IGF2R* microsatellite mutations might be important in the development of lobular cancer, although this has not been confirmed to date.

Does *M6P/IGF2R* meet all the criteria for a *bona fide* tumor suppressor gene?

In general, tumor suppressor genes encode negative regulators of cellular proliferation or promoters of cell death or differentiation. When the normal functions of tumor suppressor genes are disrupted by somatic mutation, the cell is relieved of critical regulatory signals and unrestrained proliferation or survival ensues. For a gene to be defined as a tumor suppressor from a functional rather than genetic standpoint, at least one and preferably more than one of the following observations are required: a) the *in vivo* "malignant potential" of a malignant cell line mutant for the gene in question is suppressed when wild-type protein is present in the cell; b) a gene knockout causes an increased rate of

tumor formation in transgenic mice; c) inactivating mutations are demonstrated in the germ-line of individuals with a cancer predisposition syndrome that are not present in normal family members.

At this point, M6P/IGF2R does not meet any of these operational definitions. In the next section we discuss experimental approaches that are being taken to establish whether if M6P/IGF2R meets any of these criteria.

Demonstrating M6P/IGF2R has the functional characteristics of a tumor suppressor gene.

Of the three criteria discussed above, suppression of tumorigenicity appears to be the most likely initial approach to the problem of demonstrating *M6P/IGF2R* is a tumor suppressor gene. However, a tumor cell line with biallelic loss of function mutation in *M6P/IGF2R* has not been identified, although several cell lines with a single mutated allele have been described. For example, the RER+ colon cancer cell line SW48 harbors a monoallelic poly $(G)_8$ microsatellite mutation. The remaining allele probably generates wildtype M6P/IGF2R (65) which binds IGF2 in cross-linking experiments (Schumaker, DaCosta, and Ellis, unpublished). In the absence of a null *M6P/IGF2R* cell line, a potential source of *M6P/IGF2R*-null cells are mouse embryonic fibroblasts (MEF) from a transgenic *M6P/IGF2R*-null mouse. However, MEFs are not ideal for tumor suppression experiments, since loss of the gene was not the result of a somatic mutation that was selected for during tumorigenesis. In addition, fibroblasts are of mesenchymal origin and therefore a poor model for transformation events in epithelial cells.

Despite these difficulties, there has been some success in observing a growth regulatory role for M6P/IGF2R. Kang and his co-workers (37) reported the preliminary result that overexpression of M6P/IGF2R in a retinoid-resistant human promyelocytic cell line led to RA-induced growth inhibition and apoptosis. In addition, our laboratory recently demonstrated that overexpression of M6P/IGF2R decreased IGF2-dependent proliferation of MCF7 breast cancer cells (22).

Monitoring tumor formation in transgenic *m6p/igf2r* knock-out mice would be invaluable. However, mice null for *m6p/igf2r* die perinatally. Perinatal death would not be problematic if mice heterozygous for the *m6p/igf2r* knock-out allele could be observed, since one would anticipate an increased rate of tumor formation in mice with this genotype as a result of a congenital "first hit". However, the paternal *m6p/igf2r* is imprinted in mice, with the result that mice *naturally* exhibit

monoallelic M6P/IGF2R expression from the maternal allele. Because of this mechanism, when a knock-out *m6p/igf2r* allele is inherited through the maternal germ line, the lethal phenotype is observed since the paternal M6P/IGF2R allele is silent (66). Meeting tumor suppressor definitions for imprinted genes is therefore highly problematic. One approach to circumventing the problem of perinatal mortality would be the development of an "inducible" *m6p/igf2r* knock-out mouse. In this approach, a gene can be deleted in a tissue after organogenesis has been completed. However, these approaches are complex, and so far an inducible *m6p/igf2r* knock-out has not been described.

Interestingly, unlike the situation in mice, *M6P/IGF2R* is expressed from both alleles in adult humans (67, 68). However, imprinting may persist during embryogenesis. Repression of expression from the paternal *M6P/IGF2R* allele has been detected in two of fourteen informative fetuses in one study, and it has been speculated that embryonic human *M6P/IGF2R* imprinting is a polymorphic trait (69). Interestingly, partial repression of the paternal *M6P/IGF2R* allele may occur in 50% of informative cases of Wilms' tumor, a familial childhood kidney malignancy that highly overexpresses IGF2 (70). These observations provoke the speculation that individuals with embryonic *M6P/IGF2R* imprinting might be prone to the development of embryonic tumors in which the imprinting pattern has persisted. For imprinted tumor suppressor genes, this "gain of imprinting" may be an important mechanism for inactivation.

As the *M6P/IGF2R* sequence is examined in tissues and cell lines from a number of sources, an increasing number of polymorphisms are being identified. Many of these are likely to be silent; however, if any were associated with a decrease in function, cancer predisposition might be a consequence. For this theory to be pursued, a robust set of *in vitro* assays that address multiple functions of M6P/IGF2R is a priority. Somatic mutations and germ line polymorphisms could then be examined in these assays to determine an effect on M6P/IGF2R function.

Conclusion

We conclude by stating that the case for M6P/IGF2R as tumor suppressor gene is unproven. The genetic evidence is strong, but the mutations described have not been shown to contribute to the carcinogenic process. In addition to functional experiments, further mutation screens are justified, and

LOH mapping exercises should be completed to exclude the possibility that adjacent tumor suppressor genes in 6q25-27 contribute to the rate of LOH rate at this locus in some malignancies. Finally, as our knowledge of *M6P/IGF2R* polymorphisms develops, we should explore those that lead to significant amino acid substitutions to determine the effect on M6P/IGF2R function. The recent characterization of the complete genomic structure of *M6P/IGF2R* (58) will no doubt facilitate these endeavors.

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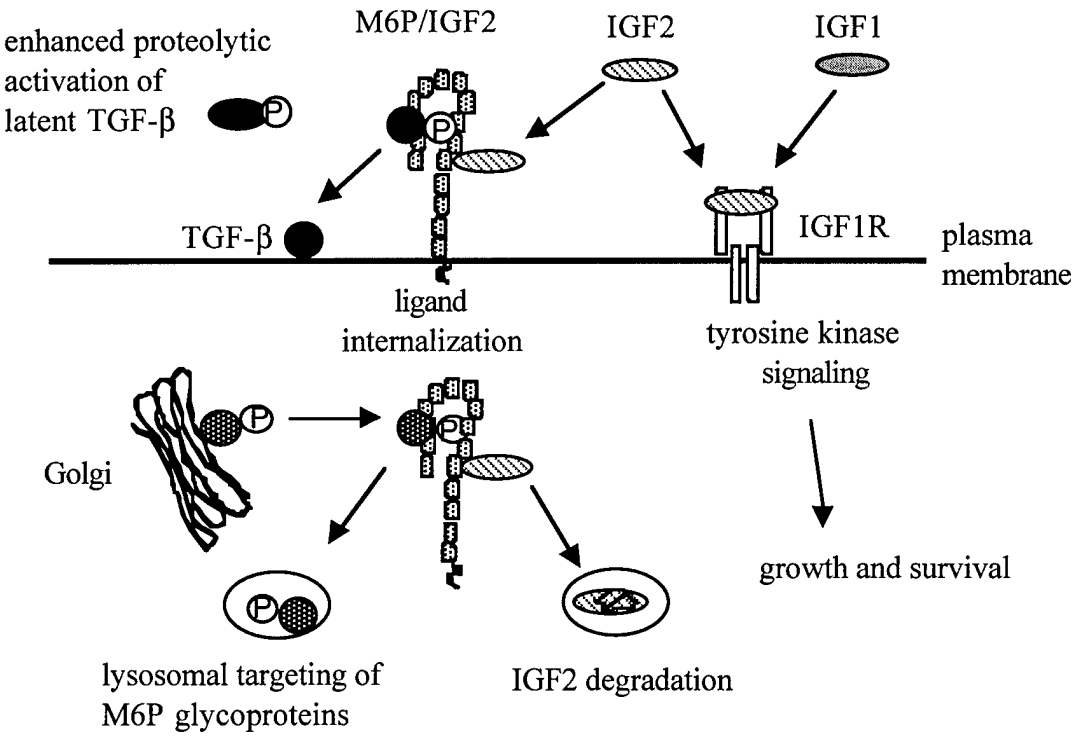

Figure 1. Summary of M6P/IGF2R function

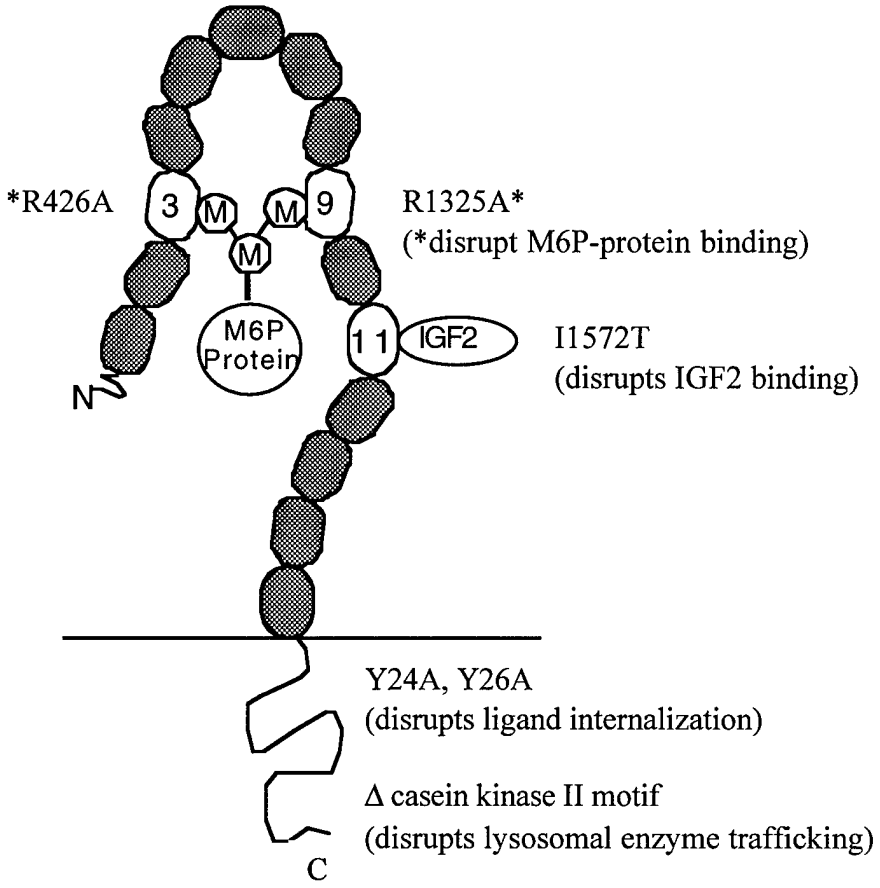

Figure 2. Site-directed mutations that define functional domains of M6P/IGF2R

Table II. LOH frequencies and mutations detected in various tumor types.

Tumor Type	LOH frequency	Mutation frequency	Mutation described	Tumor features	Ref
Breast	33% (7/21)			invasive	(1)
	26% (5/19)	40% (2/5)	Q1449H P2379T	DCIS DCIS	(1)
	22% (4/18)			DCIS	(53)
Endometrial		15% (4/26)	4089MS[a] del/insG	RER+	(6)
		14% (2/14)	4089MS del/insG	RER+	(62)
Gastrointestinal		13% (12/92)	4089MS del/insG (11) 6169MS del/ins CT (1)	RER+ RER+	(5)
		13% (4/30)	4089MS del/insG	RER+	(6)
		25% (7/28)	4089MS del/insG	RER+	(8)
Glioma		25% (1/4)	4089MS del/insG	RER+	(7)
Hepatocellular	64% (14/22)	25%	S2023STOP (1) G1449V (2)[b] G1464E (1)	Non-cirrh. Non-cirrh. Non-cirrh.	(2,3)
	61% (11/18)	55% (6/11)	C1262S (1) 4089MS del/insG (3) G1449V (2)	Cirrh. Cirrh.	(4)
Lung	52% (13/25)				(55)
Prostate	31% (5/16)				(56)

[a] MS-microsatellite
[b] No LOH detected at the *M6P/IGF2R* locus in one of these samples.

Table I. M6P/IGF2R ligands and consequences of loss receptor function

Ligands	**Function of interaction**	**Result of M6P/IGF2R loss**	**Possible consequences of M6P/IGF2R loss**
M6P-proteins	Lysosomal proteins trafficking	Misrouting/secretion of proteolytic enzymes	↑ invasion,metastasis
IGF2	IGF2 degradation	↑ IGF2 concentration	↑ proliferation ↓ apoptosis
LTGF-β	TGF-β activation	↓ active TGF-β	↑ proliferation
uPAR	TGF-β activation	↓ active TGF-β	↑ proliferation
Plasminogen	TGF-β activation	↓ active TGF-β	↑ proliferation
Retinoic acid	Enhance M6P/IGF2R function ?	?	?
Proliferin	?	?	?
LIF	?	?	?